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Award Number: DAMD17-99-1-9557

TITLE: Inflammatory Response and Oxidative Stress in the
         Degeneration of Dopamine Neurons in Parkinson's Disease

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REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual (1 Jul 01 - 1 Jul 02)

4. TITLE AND SUBTITLE
Inflammatory Response and Oxidative Stress in the Degeneration of Dopamine Neurons in Parkinson's Disease

5. FUNDING NUMBERS
DAMD17-99-1-9557

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

20021118 051

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
The experiments performed during the third grant period were an extension of the 2nd year and were designed to provide more insights in the role of arachidonic acid release and metabolism in the oxidative stress-induced cell death caused by depletion of GSH. In addition we examined the time course of free radical generation in cells depleted of GSH. Our studies using primary mesencephalic cultures provided the following information: (1) Using selective inhibitors we showed that the release of arachidonic acid occurs early in the period of GSH depletion and depends on the activation of PLA_2. (2) We showed that inhibition of PLA_2 activity protects fully from damage only if applied early in the course of GSH depletion, while inhibition of arachidonic acid metabolism is protective at any time prior to cell death. This suggests that products of the metabolism of arachidonic acid are the major cause of toxicity. (3) We confirmed that products of arachidonic acid metabolism are very toxic particularly when GSH is depleted. (3) We demonstrated that hydrogen peroxide and other reactive oxygen species (ROS) accumulate in detectable amounts only a few hours prior to cell death. Accumulation begins within mitochondria and later involves processes and cell bodies. Anti-oxidants are protective when applied anytime following damage.

14. SUBJECT TERMS
Parkinson, dopamine neuron, neurotoxin, glutathione, oxidative stress, cytokines

15. NUMBER OF PAGES
28

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Parkinson's disease is a progressive neurodegenerative disorder afflicting primarily the older population. The major neuropathological feature of Parkinson's disease is the degeneration of dopamine neurons in the substantia nigra pars compacta, which results in depletion of the neurotransmitter dopamine and loss of motor control. Our research goal is to understand the cause for the degeneration of dopamine neurons in Parkinson's disease. Oxidative stress is thought to play a role in the pathogenesis of Parkinson's disease and it has been proposed that the loss of the antioxidant glutathione (GSH) may be an early event in the development of the disorder. More recent evidence has indicated the presence of activated glial cells and signs of inflammation in the substantia nigra of Parkinson's disease patients. Our previous studies demonstrated that depletion of GSH in neuronal cultures causes cell death which is mediated by glial cells. The possibility that GSH depletion is linked to glial activation, secretion of cytokines and cell death was a focus of our studies. Two approaches were used to address this issue. First we activated glial cells in mesencephalic cultures with lipopolysaccharide (LPS) to determine the effect on GSH depletion. Contrary to our expectations, glial activation protected cells from GSH depletion-induced toxicity. The protection appeared to be due to the up-regulation of superoxide dismutase by LPS. This study, now in press (Kramer et al., 2002), indicated the significance of the superoxide radical in the toxicity caused by GSH depletion and the importance of the antioxidant enzyme superoxide dismutase for neuroprotection, information that may lead to better therapies for the treatment of Parkinson's disease. We also examined to whether anti-inflammatory agents, such as aspirin, could protect from GSH depletion. The results from these experiments were negative, although the pro-inflammatory cytokine interleukin-1β, which is secreted by activated glia, was very toxic to GSH-depleted cells, suggesting that although not

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directly linked, inflammation could increase the damage in cells depleted of GSH. The focus of our present studies is to understand the events which occur during the course of GSH depletion and their association with arachidonic acid, oxygen free radicals and cell death.

BODY

The research proposed in the statement of work for the third year of the grant period concerned the "*assessment of hydrogen peroxide formation and its role in cell death during GSH depletion*" through the use of the fluorescent dye indicator of peroxide and other oxygen free radiacls dichlorofluorescein diacetate.

During this year we also completed and extended work from the previous year concerning the role of arachidonic acid and its metabolites in the cell death caused by GSH depletion. Following is a description of the most significant findings during this year.

Restoration of GSH levels prevents BSO toxicity.

Although treatment with the GSH synthesis inhibitor l-buthionine sulfoximine (BSO) has been used extensively to cause depletion of GSH, a direct correlation between BSO-induced GSH depletion and cell death has not been demonstrated experimentally. To confirm that depletion of GSH is the primary cause of BSO toxicity, we treated mesencephalic cultures with GSH-monoethyl ether (GSH-EE), a GSH delivery agent that restores GSH levels (Meister, 1991). Figure 1A (in appendix) shows that in the presence of 1 mM GSH-EE the toxicity of 50 or 100 μM BSO was completely prevented. The protection was accompanied by an increase in GSH to levels above control values (Fig. 1B). GSH-EE also increased the levels of GSH in cultures not treated with BSO (Fig. 1B).

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PLA$_2$-dependent release of arachidonic acid increases during GSH depletion.

We completed studies examining whether GSH depletion promotes the PLA$_2$-dependent release of arachidonic acid. Membrane phospholipids were labeled with [^{3}H]arachidonic acid before treating the cultures with BSO. Spontaneous release of arachidonic acid was measured 24 h after BSO treatment, a time point when there is severe GSH depletion without cell death (Mytilineou et al., 1999). A significant increase in arachidonic acid release was observed in the BSO treated cultures, which could be prevented by the specific cPLA$_2$ inhibitor MAFP, suggesting the involvement of cPLA$_2$ in the release of arachidonic acid by BSO (Fig. 2A). Other PLA$_2$ inhibitors such as mepacrine also prevented arachidonic acid release. MTT assay showed no decrease in cell viability at this time point (Fig. 2B). These studies confirm that when GSH is depleted increased levels of arachidonic acid are released through the activation of PLA$_2$. Studies described in the previous progress report indicate that excess arachidonic acid is very toxic to GSH depleted cells.

PLA$_2$ and LOX Inhibitors Prevent BSO Toxicity; Time Course of Protection

These experiments were also a continuation of studies initiated in the last year. We showed that inhibition of cPLA$_2$ activity by the specific inhibitors ATK and MAFP prevented the toxicity of BSO in mesencephalic cultures. Also, in agreement with previous studies (Li et al., 1997; Mytilineou et al., 1999), NDGA, a non-specific LOX inhibitor and biacalein, a specific 12-LOX inhibitor, prevented BSO induced damage.

Because the increase in arachidonic acid release begins relatively early in the course of GSH depletion, we sought to determine the point in time beyond which inhibition of cPLA$_2$ or

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LOX could no longer protect from BSO toxicity. Inhibitors of PLA_2 activity were added to the cultures either at the time of exposure to BSO, or 24 and 30 and 48 hr after the beginning of BSO treatment. All groups were analyzed for cell survival at 72 hr after beginning of BSO treatment (Fig. 3). Both PLA_2 and LOX inhibitors were able to protect from toxicity, when added at the same time with BSO. However, although LOX inhibitors protected fully from toxicity even when applied 48 hr after the beginning of BSO treatment, the effectiveness of PLA_2 inhibition declined steadily. We also examined the time course of protection by the antioxidant ascorbic acid, which was previously shown to protect from BSO toxicity (Mytilineou et al., 1999). Like inhibition of LOX, ascorbic acid was able to fully protect from GSH depletion even when applied just prior to the beginning of cell death. These data indicate that the oxidative stress, which at least in part arises from the metabolism of arachidonic acid, is the main cause of cell death. We have previously reported that deprenyl, a monoamine oxidase inhibitor used in the treatment of Parkinson's disease, protects cells from GSH depletion (Mytilineou et al., 1999). We examined the time course of deprenyl neuroprotection and found that early exposure is needed for protection (Fig. 4). Although the mechanism of deprenyl neuroprotection is not understood presently, new synthesis of proteins is suggested as a mechanism of action (Tatton et al., 2002). Based on our finding that up-regulation of superoxide dismutase protects from GSH depletion (Kramer et al., 2002), we examined whether deprenyl increased superoxide dismutase activity or protein. No increase in superoxide dismutase was observed, suggesting that deprenyl operates through a different, as yet unknown, mechanism.

Reactive Oxygen Species (ROS) Accumulation in the Course of GSH Depletion

We examined the intracellular accumulation of ROS in the course of GSH depletion by loading the cells with dichlorofluorescein diacetate (DCF), which is converted to a fluorescent derivative by ROS. Cultures were treated with 50 μM BSO and then exposed to DCF at 4, 8, 24, 30 and 48 h later and observed under a fluorescent microscope. No significant increases in fluorescence could be observed in the cultures up to 30 h post treatment. High intensity fluorescence began accumulating almost simultaneously with the appearance of damaged cells (Fig. 5). Fluorescence appeared initially within well-defined organelles resembling mitochondria (Fig. 5, inset) and in cell processes and eventually filled the entire cell. Some cells appeared ballooned and detaching from the culture dish. These data are in line with the course of neuroprotection by the inhibitors of LOX and ascorbic acid and support the notion that a critical concentration of ROS is the cause of cell death.

KEY RESEARCH ACCOMPLISHMENTS:

During the period of support by the US Army we have been able to identify events that lead to cell death when cellular glutathione is depleted, a condition present in the substantia nigra in Parkinson's disease. Following is the course of events based on the studies which have been completed at present.

- Depletion of glutathione induces activation of PLA$_2$ and increased release of arachidonic acid.

- Increased activity of LOX, which has been shown to occur as a result of GSH depletion, increases the metabolism of arachidonic acid by LOX, which generates toxic ROS.

- An increase in the release of arachidonic acid begins early during the course of GSH depletion, but the cellular levels of ROS remain low for several hours, possibly through the action of alternative antioxidant mechanisms

- Cell death occurs shortly after a critical amount of ROS accumulates within the cells.

- It is possible to prevent cell death late during the course of GSH depletion by preventing the metabolism of arachidonic acid or by providing additional antioxidant defenses.

REPORTABLE OUTCOMES:

Kramer, BC, Yabut, J A, Cheong, J, JnoBaptiste, R, Robakis, T, Olanow, CW and Mytilineou, C. (2002) Lipopolysaccharide prevents cell death caused by glutathione depletion: Possible mechanisms of protection. Neuroscience, *in press*.

Kramer, BC, Yabut, JA, Cheong, J, JnoBaptiste, R, Robakis, T, Olanow, WC and Mytilineou, C. Contribution of arachidonic acid and its lipoxygenase metabolites to the neuronal toxicity of glutathione depletion (in preparation)

Yabut, JA, Kramer, BC and Mytilineou, C. Lipopolysaccharide treatment protects mesencephalic cultures from glutathioe depletion. Society for Neurosciences Abstracts, 2001.

CONCLUSIONS:

One of the early events in Parkinson's disease is the loss of GSH in the substantia nigra, which may be related to the pathogenesis of the disorder. Our research during the past three years has provided useful information concerning the survival of neurons subjected to oxidative stress due

to GSH depletion. We have shown that the depletion of GSH causes PLA$_2$-dependent release of arachidonic acid, which causes increased free radical production during its metabolism by lipoxygenase. The excess arachidonic acid metabolism when coupled with the loss of GSH creates oxidative stress beyond the antioxidant capacity of the cells and results in cell death. However, we have shown that there are ways to prevent cell death, even when GSH levels are severely depleted, by providing additional antioxidants, such as ascorbic acid and superoxide dismutase.

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Kramer BC, Yabut JA, Cheong J, Jnobaptiste R, Robakis T, Olanow CW and Mytilineou C (2002) Lipopolysaccharide prevents cell death caused by glutathione depletion: Possible mechanisms of protection. *Neuroscience* (in press):.

Li Y, Maher P and Schubert D (1997) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. *Neuron* 19:453-463.

Meister A (1991) Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacol Ther* 51:155-194.

Mytilineou C, Kokotos Leonardi ET, Kramer BC, Jamindar T and Olanow CW (1999) Glial cells mediate toxicity in glutathione-depleted mesencephalic cultures. *J Neurochem* 73:112-119.

Tatton WG, Chalmers-Redman RM, Ju WJ, Mammen M, Carlile GW, Pong AW and Tatton NA (2002) Propargylamines induce antiapoptotic new protein synthesis in serum- and nerve growth factor (NGF)-withdrawn, NGF-differentiated PC-12 cells. *J Pharmacol Exp Ther* 301:753-764.

APPENDICES:

Figures 1 - 5.
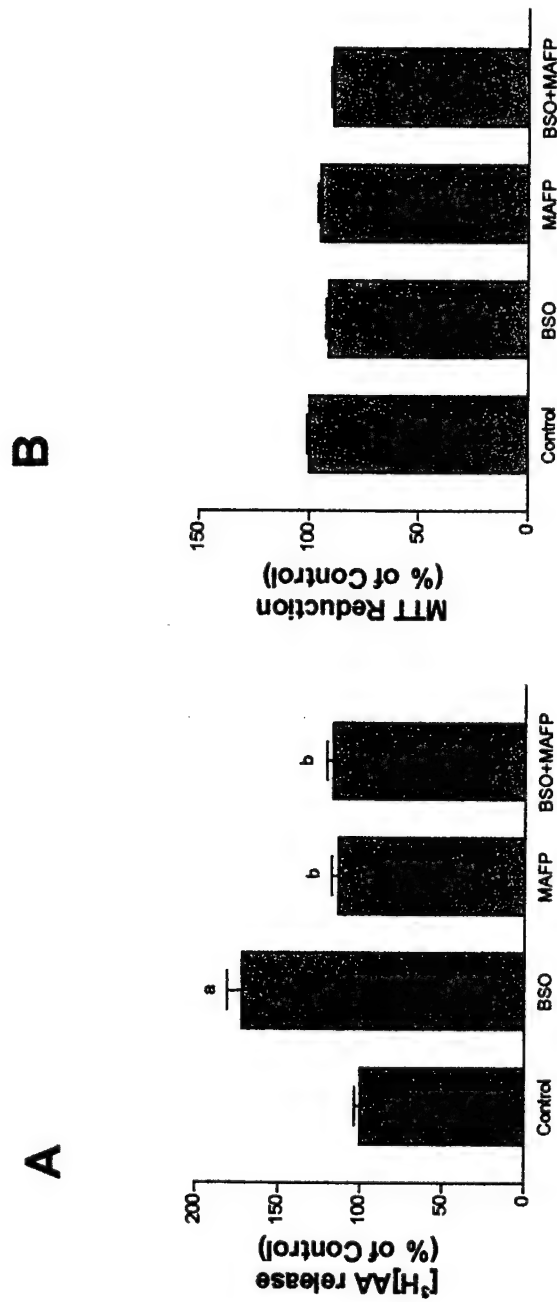
Manuscript in press in Neuroscience (2002)

Figure 1

A

a: differs from control p<0.001 (N=4/group)

B

a: differs from control p<0.001; b: differs from BSO p<0.001 (N=6/group)

Mesencephalic cultures were treated with 1mM GSH-ethyl ester and then exposed to 50 or 100 µM BSO for 48 hr. Toxicity (A) was measured with the MTT assay 48 hr after BSO treatment. Measurement of GSH levels (B) showed recovery above control levels after treatment with GSH-ethyl ester.
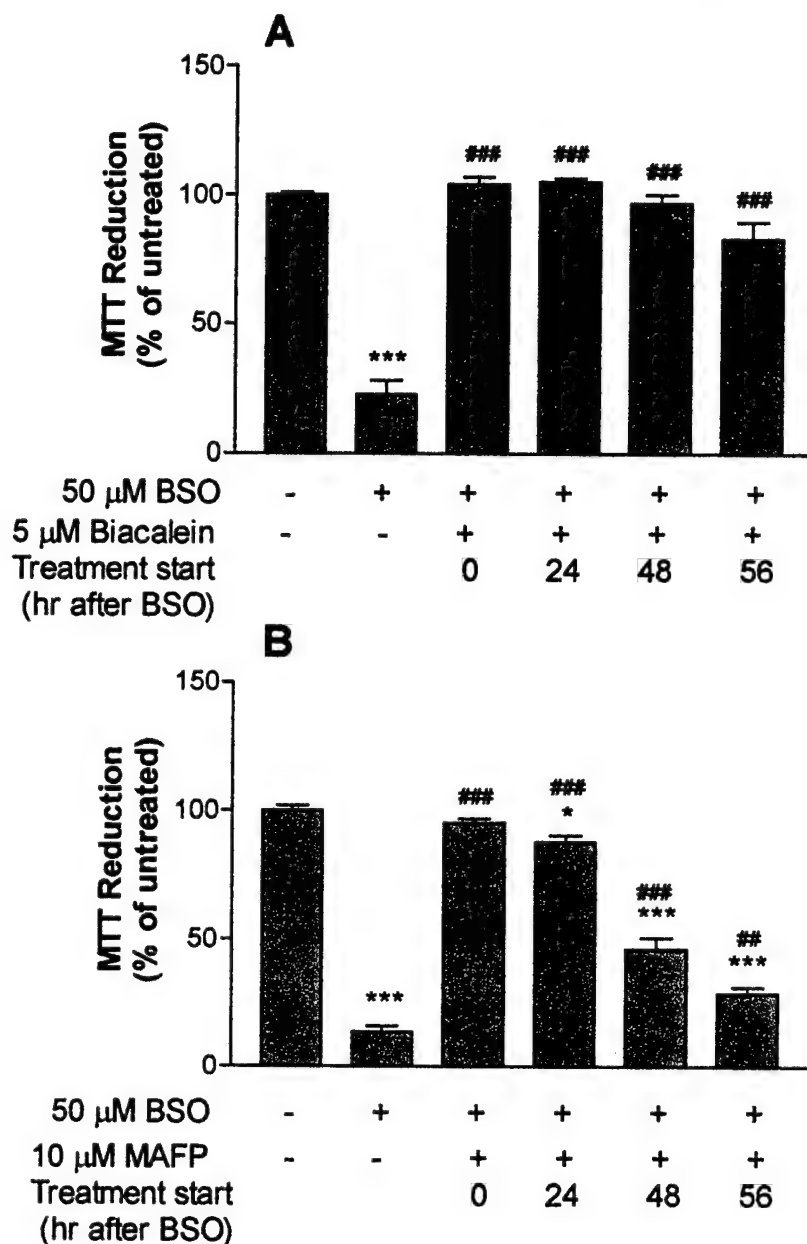
Figure 2

A

[³H]AA release (% of Control) — y-axis: 200, 150, 100, 50, 0

x-axis categories: Control, BSO, MAFP, BSO+MAFP

(labels on bars: a, b, b)

a:differs from control p<0.001; b:differs from BSO p<0.001

B

MTT Reduction (% of Control) — y-axis: 150, 100, 50, 0

x-axis categories: Control, BSO, MAFP, BSO+MAFP

A. Mesencephalic cultures were exposed to 0.5 µCi/ml [³H]arachidonic acid for 24 h to label membrane phospholipids. They were washed and treated with 50 µM BSO for an additional 24 h. Aftr a 10 min exposure to thimeroal to inhibit arachidonic acid reacylation, they were incubated with fresh buffer for 1 hr and the radioactivity released in the mdium was measured in a scintillation counter. There was increased release of arachidonic acid in the BSO treated cultures, which was prevented by treatment with the PLA₂ inhibitor MAFP.

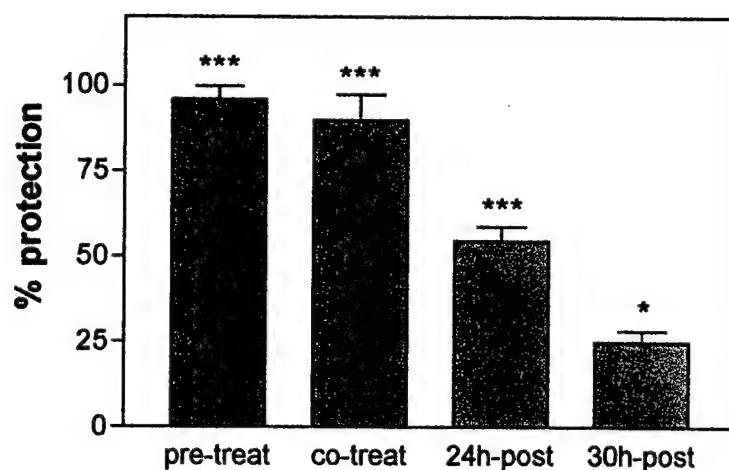B. Cultures treated as in were tested for cell viability with the MTT assay.

Figure 3

Cultures were treated with 50 μM BSO for 72 hr. The LOX inhibitor biacalein (5 μM) (A) or the PLA$_2$ inhibitor MAFP (10 μM) (B) were added at the same time with BSO (0), or 24, 48 and 56 hr later. ***Difers from Control $p<0.001$; ###differs from BSO $p<0.001$; ##$p<0.01$

Figure 4

Time course of protection by deprenyl

Groups of cultures were treated with 50 μM deprenyl 24h prior to treatment with 50 μM BSO, at the same time with BSO or 24 or 30h post treatment. LDH assay for cell viability was performed 48h after the beginning of BSO treatment. In the pre-treatment group, deprenyl was also added with BSO. ***Differs from BSO alone $p<0.001$; *$p<0.05$. Anova followed by Tukey test.

Figure 5

Control   **BSO 48h**
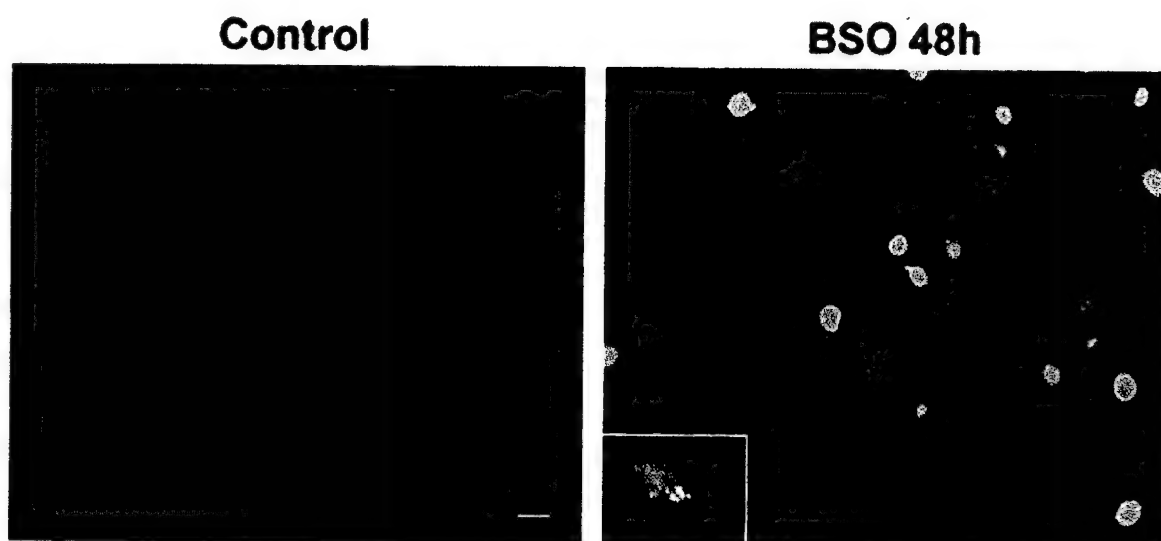
Controls and cultures treated with 50 uM BSO were exposed to DCF for
15 min 48h after BSO. Intense fluorescence indicating the presence of ROS
was observed at this time point incultures treated with BSO. Inset: higher
magnification showing fluorescence in well defined cytoplasmic organelles,
resembling mitochondria.

LIPOPOLYSACCHARIDE PREVENTS CELL DEATH CAUSED BY GLUTATHIONE DEPLETION: POSSIBLE MECHANISMS OF PROTECTION

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Abstract—Glutathione is an important cellular antioxidant present at high concentrations in the brain. We have previously demonstrated that depletion of glutathione in mesencephalic cultures results in cell death and that the presence of glia is necessary for the expression of toxicity. Cell death following glutathione depletion can be prevented by inhibition of lipoxygenase activity, implicating arachidonic acid metabolism in the toxic events. In this study we examined the effect of glial activation, known to cause secretion of cytokines and release of arachidonic acid, on the toxicity induced by glutathione depletion. Our data show that treatment with the endotoxin lipopolysaccharide activated glial cells in mesencephalic cultures, increased interleukin-1β in microglia and caused depletion of glutathione. The overall effect of lipopolysaccharide treatment, however, was protection from damage caused by glutathione depletion. Addition of cytokines or growth factors, normally secreted by activated glia, did not modify L-buthionine sulfoximine toxicity, although basic fibroblast growth factor provided some protection. A large increase in the protein content and the activity of Mn–superoxide dismutase, observed after lipopolysaccharide treatment, may indicate a role for this mitochondrial antioxidant enzyme in the protective effect of lipopolysaccharide. This was supported by the suppression of toxicity by exogenous superoxide dismutase. Our data suggest that superoxide contributes to the damage caused by glutathione depletion and that up-regulation of superoxide dismutase may offer protection in neurodegenerative diseases associated with glutathione depletion and oxidative stress. © 2002 Published by Elsevier Science Ltd on behalf of IBRO.

Key words: L-buthionine sulfoximine, mesencephalic cultures, superoxide dismutase, oxidative stress, Parkinson's disease.

Oxidative stress is believed to contribute to the degeneration of dopamine neurons in Parkinson's disease (PD). Hydrogen peroxide (H_2O_2) is a major oxidative species produced normally within neurons during respiration. Within the dopamine neurons H_2O_2 is also formed during the metabolism of dopamine by monoamine oxidase. The high-energy requirements of brain function coupled with the metabolism of dopamine could result in concentrations of H_2O_2 sufficient to cause oxidative stress in

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Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; BSO, L-buthionine sulfoximine; EDTA, ethylenediaminetetra-acetate; GFAP, glial fibrillary acidic protein; GSH, reduced glutathione; IGF, insulin-like growth factor; IL-1β, interleukin-1β; INF-γ, interferon-γ; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAP, microtubule associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PD, Parkinson's disease; PLA₂, phospholipase A_2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TGF-β, transforming growth factor-β; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-α; TTBS, 20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween 20, pH 7.6.

dopamine neurons (Cohen and Kesler, 1999). Glutathione (GSH), an important soluble antioxidant in the brain, detoxifies H_2O_2 and lipid hydroperoxides (Meister, 1991). GSH is depleted in the substantia nigra in PD (Perry et al., 1982; Sofic et al., 1992) and it has been suggested that this loss may be an early event in its pathogenesis (Dexter et al., 1994).

Inhibition of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis, by L-buthionine sulfoximine (BSO) causes cell death in primary neuronal cultures and cell lines (Li et al., 1997; Mytilineou et al., 1999; Wullner et al., 1999). Inhibition of lipoxygenase activity prevents cell death, implicating arachidonic acid metabolism in the toxicity of GSH depletion (Li et al., 1997; Mytilineou et al., 1999). We have recently shown that damage from GSH depletion becomes greater in mesencephalic cultures with increased glia to neuron ratios (Mytilineou et al., 1999). The contribution of glia to the toxicity of GSH depletion is not well understood and, in view of the well-known neuroprotective role of astrocytes (Desagher et al., 1996; O'Malley et al., 1994; Takeshima et al., 1994; Wilson, 1997), such an effect may appear counter-intuitive. Several findings, however, could implicate astrocytes in arachidonic acid-mediated toxicity in primary cell cultures: (1) Among brain cells, astrocytes are exclusively responsible for the synthesis of arachidonic acid (Katsuki and Okuda, 1995). (2) Neurons depend on astrocytes for arachidonic acid

transport, because they cannot perform the fatty acid desaturation steps necessary for its synthesis (Katsuki and Okuda, 1995; Moore et al., 1991). (3) The cytosolic form of phospholipase A$_2$ (PLA$_2$), the enzyme primarily responsible for the release of arachidonic acid in brain, is present within astrocytes (Stephenson et al., 1999; Stephenson et al., 1994) and microglial cells (Clemens et al., 1996).

Activation of glial cells causes secretion of cytokines, increases PLA$_2$ activity (Oka and Arita, 1991) and induces release of arachidonic acid in astrocytes (Minghetti and Levi, 1998; Stella et al., 1997) and microglia (Minghetti and Levi, 1998). Theoretically, glial activation combined with GSH depletion, which also increases lipoxygenase activity (Li et al., 1997; Shornick and Holtzman, 1993), should result in excess free radical generation and create additional oxidative challenge to GSH depleted cells.

To study the role of glial activation in the toxicity of GSH depletion, we exposed mesencephalic cultures to a bacterial endotoxin (lipopolysaccharide; LPS), prior to treatment with BSO. We show that LPS activated microglia and astrocytes, reduced the levels of GSH and increased the cytokine interleukin-1β (IL-1β), which is toxic to GSH depleted cells. However, the overall effect of LPS was a significant protection from the toxicity of GSH depletion. Our data suggest that the up-regulation of Mn-dependent superoxide dismutase (SOD), the mitochondrial enzyme responsible for detoxification of superoxide (O$_2^-$) by LPS, may play a significant role in the LPS-induced protection.

EXPERIMENTAL PROCEDURES

Materials

Pregnant Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY, USA). MEM was purchased from Gibco-Life Technologies (Grand Island, NY, USA), horse serum from Gemini (Calabasas, CA, USA) and NU® serum from Collaborative Biomedical Products (Bedford, MA, USA). LPS (from *Escherichia coli* serotype 026:B6 and 0111:B4) and other chemicals were obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies to glial fibrillary acidic protein (GFAP) were purchased from Sigma (St. Louis, MO, USA) and to tyrosine hydroxylase (TH) from Chemicon (Temecula, CA, USA). Monoclonal OX-42 antibodies against the rat microglial surface antigen complement receptor type 3 (Mac-1) (Perry et al., 1985) were purchased from Chemicon (Temecula, CA, USA). Polyclonal antibodies to IL-1β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), to Cu/ZnSOD from Chemicon and to MnSOD from StressGen Biotechnologies (Victoria, BC, Canada).

Cell cultures

The protocols for handling animals and preparing cell cultures followed the NIH guidelines and were approved by the institutional review committee. Mesencephalic cultures were prepared from embryonic rats on the 14th day of gestation as described previously (Mytilineou et al., 1999). In brief, the mesencephalon was dissected free of meninges and collected in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The tissue was mechanically dissociated into a single cell suspension and plated in 24- or six-well plates precoated with L-polyorni-

thine (0.1 mg/ml) at a density of 300 000 cells/cm^2. The medium consisted of MEM supplemented with 2 mM glutamine, 33 mM glucose, 10% horse serum and 10% NU® serum. Treatment began on the fifth or sixth day *in vitro*, at which time the medium was changed to MEM containing only 5% NU® serum. The method of McCarthy and de Vellis (1980) was used to prepare purified astrocytes. In brief, cortical cultures prepared from newborn rats were plated in 75 cm^2 flasks at 5×10^6 cells/flask. After seven days *in vitro* the flasks were shaken overnight on a rotary shaker at 250 r.p.m. and the following day the floating cells were removed and the remaining attached astrocytes were dislodged with Versene® and plated in 24-well plates. Treatment of astrocyte cultures began 24 h after plating.

Immunocytochemistry

Cells were plated on polyornithine coated glass coverslips in 24-well plates. They were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized and blocked with 0.3% Triton X-100 and 3% bovine serum albumin (BSA) for 30 min. Primary antibodies used were: anti-GFAP (1:1000), OX-42 (1:250), anti-microtubule associated protein (MAP-2 1:500), anti-IL-1β (1:250) and anti-TH (1:1000). Cultures were exposed to the primary antibodies overnight at 4°C. Secondary antibodies conjugated to Alexa fluorescent dyes (Molecular Probes, Eugene, OR, USA) were used at a dilution of 1:1000 for 30 min. The cultures were observed with an Olympus fluorescence microscope and the images recorded with a Spot video camera.

[³H]dopamine uptake

Measurement of dopamine uptake was performed as described previously (Mytilineou et al., 1998). Cultures were rinsed with Kreb's phosphate buffer (pH 7.4) and incubated for 30 min at 37°C with the same buffer containing 0.2 mg/ml ascorbic acid and 0.5 µCi/ml [³H]dopamine (32.6 Ci/mmol; NEN, Boston, MA, USA). After rinsing, the radioactivity was extracted with 1 ml 95% ethanol, which was added to vials containing scintillation cocktail and the radioactivity measured in a scintillation spectrometer (Packard Tri-Carb 2100). Cultures treated with the neuronal dopamine uptake blocker mazindol (10 µM) were used as blanks.

Cell viability assays – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined by the MTT reduction assay, as described previously (Han et al., 1996). In brief, 50 µl of a 5 mg/ml solution of MTT was added to each cell culture well containing 0.5 ml medium. After a 1-h incubation at 37°C, the medium was carefully removed and the formazan crystals were dissolved in 1 ml isopropyl alcohol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250, Molecular Devices Corporation, Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) assay

A modification of the method by Bergmeyer et al. (1963) was used to determine LDH activity in the culture medium and the cells. Medium was collected, centrifuged to remove debris and frozen at −80°C until assay. Cells were freeze-thawed (×3) in 0.5 ml feeding medium, the medium was collected, centrifuged and the supernatant frozen at −80°C. 50 µl of supernatant and 100 µl of NADH (1.2 mg/ml H$_2$O stock) were added to 850 µl of buffer and the samples were vortex-mixed. 50 µl of feeding medium was used for blanks. Triplicate aliquots (250 µl) were placed into 96-well plates at room temperature and reaction was initiated by addition of 25 µl of sodium pyruvate (0.36 mg/ml H$_2$O stock). The rate of disappearance of NADH was measured at 340 nm using a plate reader.

GSH assay

GSH was quantified using a modification of a standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) with GSH reductase and NADPH (Tietze, 1969). In brief, the medium was carefully aspirated from the culture wells, 300 μl of 0.4 N perchloric acid was added and the plates were kept on ice for 30 min. The perchloric acid was then collected and stored at −80°C until assayed. Both oxidized (GSSG) and reduced (GSH) forms of glutathione are measured with this assay. However, because of the small amounts of GSSG present in mesencephalic cultures (∼5% of total; Mytilineou et al., 1993), the values obtained were considered to represent GSH content.

The tissue attached to the bottom of the wells after removal of the PCA was dissolved in equal volumes of 20% SDS (sodium dodecyl sulfate) and 0.5 N NaOH and used for protein determination according to the method of Lowry (Lowry et al., 1951) with BSA as a standard.

SOD assay

Cells plated in six-well plates were collected in cold PBS (0.1 ml/well). Three to six wells were pooled for each sample. Cells were sonicated on ice, centrifuged at $4000 \times g$ for 10 min at 4°C and dialyzed overnight in PBS at 4°C (Slide-A-Lyzer dialysis cassettes, 10 K cut-off; Pierce, Rockford, IL, USA). SOD activity was assayed in 50 μl of the dialysate according to the assay developed by McCord and Fridovich (1969), modified for use with a microplate reader. In brief, 100 μl of xanthine solution (4 mM in 0.01 M NaOH) and 10 μl of cytochrome C (partially acetylated; 0.5 mg/ml) were added to 900 μl of 50 mM phosphate buffer. After the addition of 50 μl of sample, buffer for blanks or SOD for standards, triplicates of 250 μl were placed in the wells of a 96-well plate and the reaction was initiated by the addition of 25 μl/well xanthine oxidase (0.75 U/ml in 0.1 mM EDTA). The reduction rate of cytochrome c by superoxide radicals was monitored at 550 nm at 25°C for 10 min. Total SOD activity in the samples was determined from a standard curve and was corrected for protein content. MnSOD activity was determined after inhibition of the Cu/ZnSOD by a 5-min incubation with 2 mM KCN.

Catalase assay

Catalase activity was measured by a modification of the method described by Bordet et al. (2000). In brief, cells were plated in six-well plates and collected in PBS containing a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA). Three wells (0.1 ml/well) were pooled for each sample. The cells were freeze-thawed once, homogenized, centrifuged at $4000 \times g$ for 10 min and the supernatants stored frozen at −80°C until use. Cell extracts containing 50 μg protein were added to 250 μl of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.8) and the disappearance of H_2O_2 was measured at 240 nm for 2 min an 5-s intervals in a plate reader.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The dialyzed cell homogenates used for SOD assays were also used for western blotting. Samples containing 20 μg of protein were mixed with 20 μl Laemmli buffer. The proteins were resolved on a 12% SDS–PAGE (Bio-RAD, Hercules, CA, USA) and transferred to Hybond®-P PVDF membrane (Amersham Pharmacia, Piscataway, NJ, USA) for 1 h at 15 V, using a Trans-Blot Semi-Dry Transfer Cell (Bio-RAD, Hercules, CA, USA). Membranes were incubated in blocking solution (5% milk, 5% newborn-calf serum) in TTBS (20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween 20, pH 7.6) at 4°C overnight and then with polyclonal antibodies to Cu/ZnSOD or MnSOD and with monoclonal antibody to TH in blocking solution for 2 h. They were then washed with TTBS and incubated with horse-radish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (whole molecule) antibodies (ICN, Aurora, OH, USA) in blocking solution for 1 h at room temperature. After washing with TTBS the membranes were visualized with ECL Plus western blot detection system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

All data are expressed as means ± S.E.M. Significance of differences between groups was determined using one-way analysis of variance (ANOVA) with Tukey–Kramer post-test for multiple comparisons.

RESULTS

LPS causes glial activation in mesencephalic cultures

LPS, an endotoxin used extensively to induce glial activation in vivo (Castano et al., 1998) and in vitro (Bronstein et al., 1995; Jeohn et al., 1998), was added to the medium of mesencephalic cultures at day 5 in vitro. Specific markers for astrocytes and microglia were used to test for glial activation. After 72-h exposure to 10 μg/ml LPS, immunocytochemistry with antibodies to GFAP revealed an increase in the number of astrocytic processes compared with the controls (Fig. 1A, B). The number and size of microglia cells labeled with OX-42 was also increased after LPS treatment (Fig. 1C, D). We also tested for the presence of the pro-inflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α), which are secreted by activated glial cells (Benveniste, 1998). IL-1β was present exclusively in OX-42-labeled cells, indicating microglial localization of this cytokine (Fig. 1E, F). Labeling for IL-1β was weak in most microglia of control cultures (Fig. 1E). An increase in IL-1β immunoreactivity in OX-42-positive cells was observed after LPS treatment (Fig. 1F). Antibodies to TNF-α labeled both neurons and astrocytes and there was no apparent difference in intensity or distribution between control and LPS-treated cultures (not shown).

LPS provides protection against BSO-induced toxicity

To examine the effect of glial activation on GSH depletion-induced damage, cultures were pretreated with 10 μg/ml LPS for 24 h and then exposed to the GSH synthesis inhibitor, BSO, for an additional 48 h. LPS was present in the medium during BSO treatment. In agreement with our previous studies (Mytilineou et al., 1999; Mytilineou et al., 1998), BSO at 10 and 50 μM caused significant damage, as assessed by the MTT assay (Fig. 2A). Treatment with LPS significantly attenuated the BSO-induced damage (Fig. 2A). Cell viability was reduced to 42% and 9% of control values after a 48 h treatment with 10 and 50 μM BSO respectively, and LPS treatment improved survival to 65% and 56% of control with the same BSO treatment.

LPS concentrations ranging from 1 (the lowest effective concentration) to 50 μg/ml were equally protective from BSO toxicity (Fig. 2B). In the experiment presented
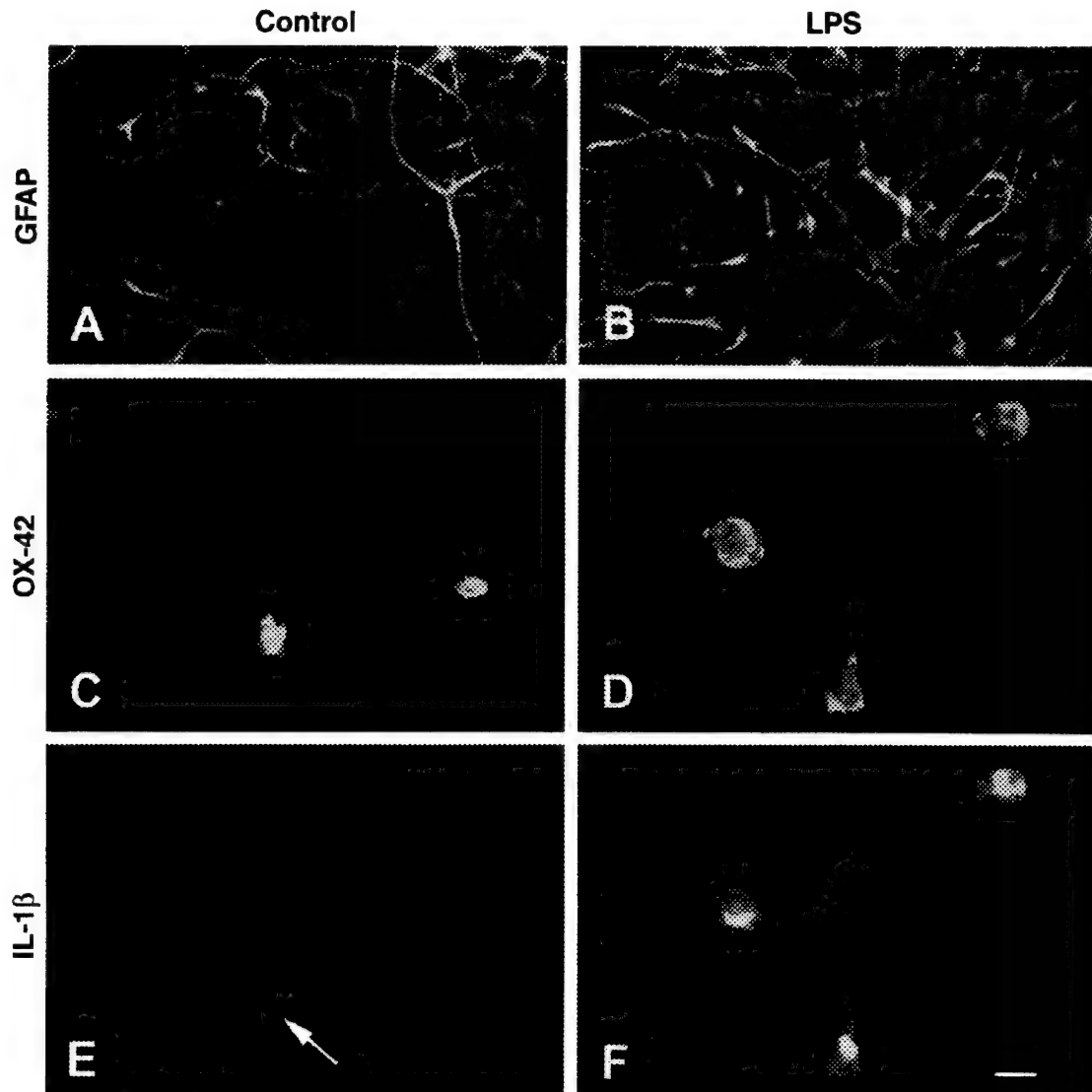
Control   **LPS**

Fig. 1. Treatment with LPS causes activation of astrocytes and microglia in mesencephalic cultures. Immunocytochemistry for GFAP (A, B), OX-42 (C, D) and IL-1β (E, F) in control cultures (A, C, E) and cultures treated with 10 μg/ml LPS for 72 h. Increased labeling of GFAP-positive astrocytes was apparent in cultures treated with LPS (compare A and B). OX-42 labeling of microglia showed an increase in the size after treatment with LPS (compare C and D). The number of microglia was also increased after LPS treatment. Panels E and F show the same fields as C and D double labeled for IL-1β. In control cultures (E), some microglia (arrow) express low levels of the cytokine. After LPS treatment (F), all OX-42-labeled cells show intense immunoreactivity for IL-1β. Scale bar = 25 μm.

in Fig. 2B, 50 μM BSO caused only moderate loss of cell viability after 48 h (47% loss) and LPS prevented this damage at all concentrations tested. Differences in the extent of toxicity caused by BSO in some experiments, are believed to be due to variations among primary cultures from different preparations.

It has been reported that LPS causes neuronal toxicity *in vitro*, which can be selective for dopamine neurons (Bronstein et al., 1995; Jeohn et al., 1998), and is sometimes achieved with ng/ml concentrations of LPS (Liu et al., 2000). To address the apparent conflict between these reports and our data, we compared the effect of LPS from *E. coli*, serotype 0111:B4 used by Liu et al

(2000), with the serotype 026:B6 used in our study. The concentrations used ranged from 0.1ng/ml to 10 μg/ml, which included the concentrations used in both studies. MTT assay to determine overall cell survival showed no detectable toxicity in our culture system at any LPS concentration (Table 1). Measurement of [^{3}H]dopamine uptake, which was used in the study by Liu et al. (2000) to assess for selective damage to dopamine neurons, also demonstrated no toxicity of LPS (Table 1). We speculate that the lack of LPS-induced damage in our experiments was likely due to the different cell culture conditions (feeding medium, cell density, etc.).
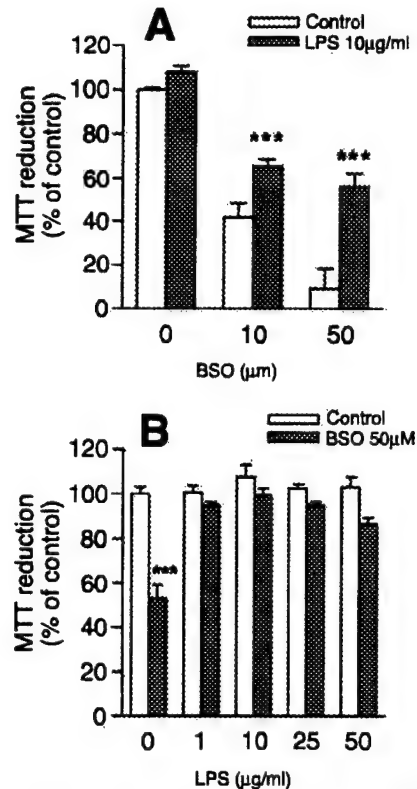
Fig. 2. LPS protects mesencephalic cultures from the toxicity of BSO treatment. (A) Cultures were exposed to 10 μg/ml LPS for 24 h and then treated with 10 or 50 μM BSO for an additional 48 h in the presence or absence of LPS. (B) Cultures treated with different concentrations of LPS for 24 h and then exposed to 50 μM BSO for 48 h. Bars show means ± S.E.M. (n = 4 per group; these experiments were repeated with similar results). ***P < 0.001 compared to the corresponding control; ANOVA followed by Tukey–Kramer multiple comparisons test.

LPS lowers GSH levels in astrocytes and mesencephalic cultures

We examined whether protection by LPS was the result of a direct effect on GSH levels. Since astrocytes,

the major glial component in mesencephalic cultures, are important regulators of neuronal GSH (Sagara et al., 1993), we also tested the effect of LPS on GSH levels in cultures enriched in astrocytes. Astrocytes were treated with 1 or 10 μg/ml LPS for 24 h and then exposed to 10 μM BSO for an additional 48 h. After BSO treatment alone the levels of GSH were reduced to 43% of control values (Fig. 3A). In cultures treated with 1 or 10 μg/ml LPS, BSO caused an even greater decrease in GSH, to 14% and 13% of control levels respectively. Treatment with LPS alone caused significant reduction in GSH levels as well (to 73% and 76% of control with 1 and 10 μg/ml LPS, respectively). Neither LPS nor BSO treatment caused any loss in the viability of astrocytes as determined with the MTT assay (results not shown).

Treatment of mixed neuronal-glial cultures with LPS also caused a significant loss of GSH, reducing the levels to 77% and 25% of control values with 1 and 10 μg/ml, respectively (Fig. 3B). Treatment with 10 μM BSO for 48 h caused severe loss of cells in cultures not exposed to LPS and the GSH levels were only about 2% of controls. However, the GSH content in the few surviving cells may actually be underestimated when expressed in nmol/per mg protein, as protein measurement includes cell debris still attached to the culture dish. GSH levels in cultures treated with 10 μM BSO and 1 μg/ml LPS were 15% of control values, while 10 μg/ml LPS decreased GSH to levels not detectable by our assay, even though no apparent damage to the cells could be observed by phase contrast microscopy.

Possible mechanisms of LPS protection

Effect of growth factors and cytokines. Activated glial cells secrete both toxic and trophic substances. We tested the effects of insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF), known to be secreted by glial cells, on the toxicity of BSO in mesencephalic cultures (Fig. 4). In these experiments BSO-induced damage was determined by measuring the amount of LDH released into the culture medium. The concentration of growth factors used was 50 or 100 ng/ml and was selected for

Table 1. The effect of LPS obtained from different *E. coli* clones on cell survival (MTT reduction) and [³H]dopamine uptake in mesencephalic cultures

LPS (ng/ml)	MTT reduction (% of control)		[³H]dopamine uptake (% of control)	
	Clone 0111:B4	Clone 026:B6	Clone 0111:B4	Clone 026:B6
0	100.0 ± 1.7	100.0 ± 1.6	100. 0 ± 3.8	100.0 ± 1.7
0.1	97.5 ± 1.5	98.1 ± 3.1	91.3 ± 1.8	99.2 ± 3.9
1	100.2 ± 0.2	98.6 ± 2.0	99.2 ± 3.4	97.8 ± 1.9
10	98.6 ± 1.9	99.7 ± 2.7	97.2 ± 1.4	93.8 ± 6.7
100	96.5 ± 0.8	92.5 ± 3.6	96.3 ± 4.4	93.1 ± 2.5
10 000	93.7 ± 0.5	98.5 ± 2.9	89.0 ± 1.4	98.0 ± 6.7

Mesencephalic cultures were exposed on the fifth day *in vitro* to LPS for 24 h and then treated again with LPS for an additional 48 h in order to duplicate the conditions used in the experiments examining the effect of LPS on the damage caused by GSH depletion. MTT assay and [³H]dopamine uptake was performed at the end of LPS treatment. ANOVA showed no significant differences in MTT reduction or [³H]dopamine uptake following LPS treatment.
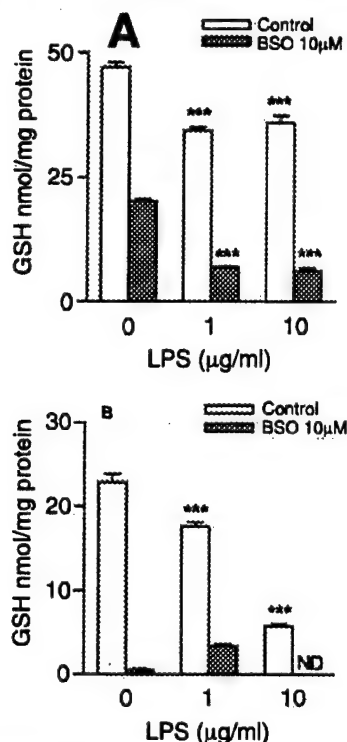
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Fig. 3. Treatment with LPS causes reduction in GSH levels in purified astrocytes and mesencephalic cultures. Astrocytes and mesencephalic cultures were exposed to 1 or 10 μg/ml LPS for 24 h and then treated with 10 μM BSO for an additional 48 h. LPS decreased GSH levels in astrocytes (A) and potentiated the depletion caused by BSO. In mesencephalic cultures (B) LPS lowered GSH content significantly, particularly at the higher concentration. Mesencephalic cultures treated with BSO had minimal cell survival and very low GSH levels. In the presence of 10 μg/ml LPS and BSO GSH levels were not detectable (ND), although there was no apparent damage to the cells. Bars show means ± S.E.M. (n = 4 per group) ***P < 0.001; ANOVA followed by Tukey–Kramer multiple comparisons test.

maximal trophic activity towards neurons (Bouvier and Mytilineou, 1995). bFGF protected mesencephalic cells from toxicity at the lower concentrations of BSO (5 and 10 μM; Fig. 4A). With 50 μM BSO the damage was extensive (64% of total LDH was released into the medium) and bFGF was unable to confer any protection. Neither TGF-β nor IGF-I had any effect on BSO toxicity (Fig. 4B, C).

A number of pro- and anti-inflammatory cytokines are secreted upon activation of glial cells (Aschner, 1998; Giulian et al., 1993; Minghetti and Levi, 1998). We tested whether the pro-inflammatory cytokines IL-1β, interferon-γ (INF-γ) or TNF-α can modify BSO-induced toxicity. As shown in Fig. 5, exposure to IL-1β significantly increased BSO toxicity, while INF-γ (50 or 100 ng/ml) and TNF-α (20 ng/ml) had no effect (results not shown). The cytokines IL-6 and IL-10 (50 or 100 ng/ml), which can have anti-inflammatory properties and may be neuroprotective, were also tested but failed to provide protection against BSO toxicity (results not shown).

Up-regulation of antioxidant enzymes. LPS has been shown to up-regulate the antioxidant enzyme SOD in glial cultures (Del Vecchio and Shaffer, 1991; Mokuno et al., 1994). We examined the effect of LPS treatment (10 μg/ml for 48 h) on SOD and catalase activity in mesencephalic cultures. Treatment with LPS caused a greater than two-fold increase in MnSOD activity, but had no significant effect on Cu/ZnSOD (Fig. 6A). Catalase activity was not altered by LPS treatment (catalase activity after treatment with 10 μg/ml LPS was 106.7 ± 6.1% of control values, n = 3/group). MnSOD protein levels were also higher after LPS treatment (Fig. 6B), with no change in Cu/ZnSOD. To test the possibility that increased levels of SOD played a role in the protection from BSO toxicity we treated cultures with BSO in the presence or absence of SOD. Cu/ZnSOD or MnSOD added to the medium at 300 or 500 U/ml, provided significant protection from BSO toxicity (Fig. 7).
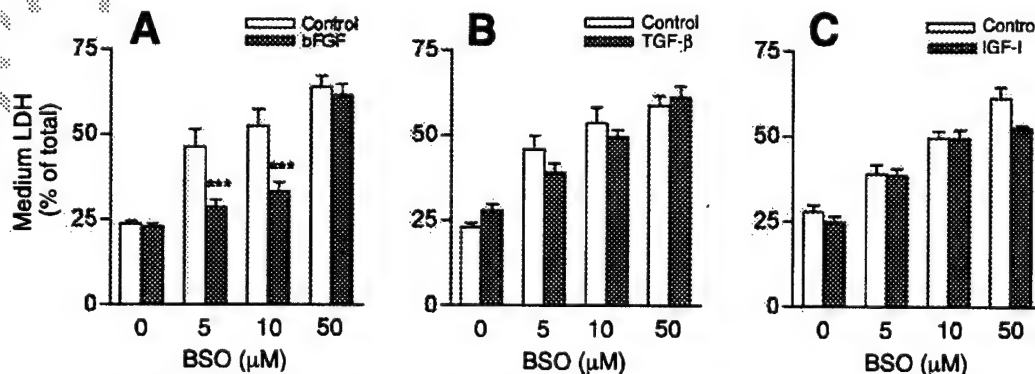

Fig. 4. The effect of growth factors on BSO toxicity. Mesencephalic cultures were treated with 50 ng/ml bFGF, TGF-β, or IGF-I for 24 h before exposure to BSO for an additional 48 h. Growth factors were present during BSO treatment. LDH released in the medium was expressed as percent of total LDH (medium+cells). Bars show means ± S.E.M. (n = 12, from three separate experiments). ***P < 0.001; ANOVA followed by Tukey–Kramer multiple comparisons test. Similar results were obtained with 100 ng/ml growth factors.
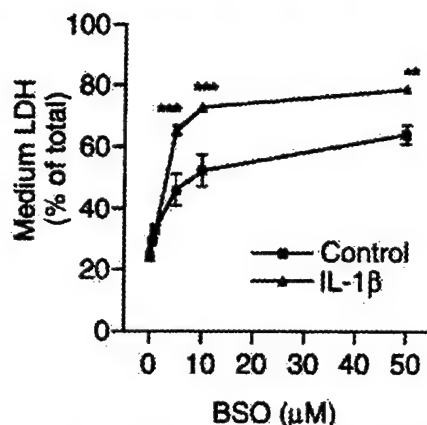
Fig. 5. The effect of IL-1β on BSO toxicity. Mesencephalic cultures were treated with 50 ng/ml IL-1β for 24 h before exposure to BSO for an additional 48 h. IL-1β was present during BSO treatment. LDH released in the medium was expressed as percent of total LDH (medium+cells). Bars show means ± S.E.M. ($n = 10$–12, from three separate experiments). ***$P < 0.001$; **$P < 0.01$; ANOVA followed by Tukey–Kramer multiple comparisons test.

The localization of Cu/ZnSOD and MnSOD in mesencephalic cultures was examined using antibodies specific for the two enzymes. Labeling for Cu/ZnSOD was diffuse and was present at varying intensities mostly within neurons (Fig. 8A–C). No difference could be observed in Cu/ZnSOD labeling between control and LPS-treated cultures (Fig. 8A, D). Labeling for MnSOD was punctate, reflecting its mitochondrial localization (Fig. 9). In control cultures, MnSOD was present primarily within neurons, as demonstrated by its co-localization with MAP-2 (Fig. 9A–C). The intensity of MnSOD labeling in the neurons did not seem affected by LPS (Fig. 9D–F), though non-neuronal labeling was substantially increased. Under control conditions, little MnSOD immunoreactivity could be found in GFAP-positive astrocytes (Fig. 9G–I). After LPS treatment, however, very intense labeling appeared within GFAP-positive astrocytes (Fig. 9J–L). The somewhat weaker MnSOD label in the neurons of the LPS-treated cultures (Fig. 9D) is due to the very high intensity of the fluorescent label in the surrounding astrocytes, which prevents accurate photographic representation.

DISCUSSION

Our study shows that LPS protects mesencephalic cultures from damage caused by the inhibition of GSH synthesis with BSO. LPS treatment caused activation of glial cells, which was confirmed by the changes in morphology and the increase in the size of microglia (Kreutzberg,
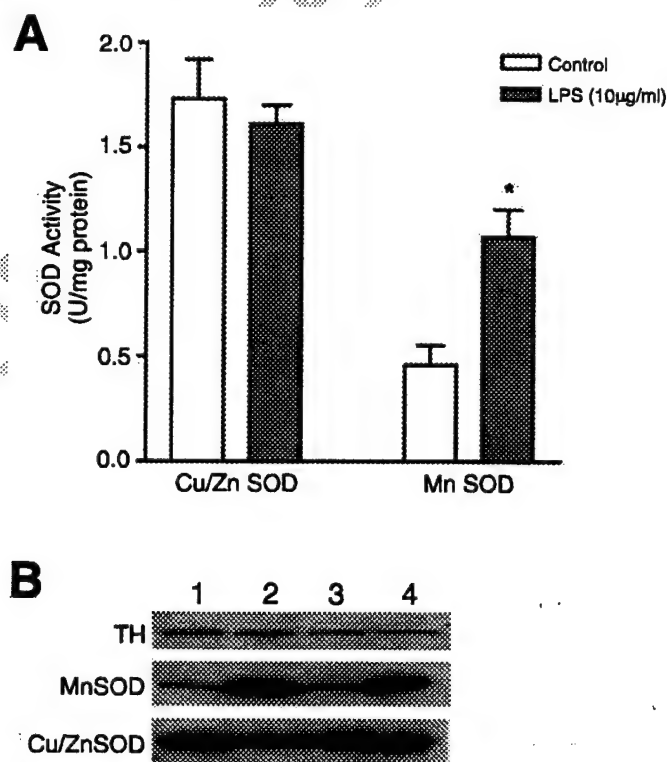

Fig. 6. LPS treatment up-regulates MnSOD in mesencephalic cultures. Cultures were treated with 10 μg/ml LPS for a total of 72 h. (A) SOD activity, measured in triplicates from pooled cells, from three separate experiments. Bars show means ± S.E.M. ($n = 3$). *$P < 0.05$ Student's t-test. (B) Western blots of lysates from control (lanes 1 and 3) and LPS-treated (lanes 2 and 4) cultures from two independent experiments. Antibodies to TH and MnSOD were applied to the same blot. Separate blots from the same lysates were used for Cu/ZnSOD.
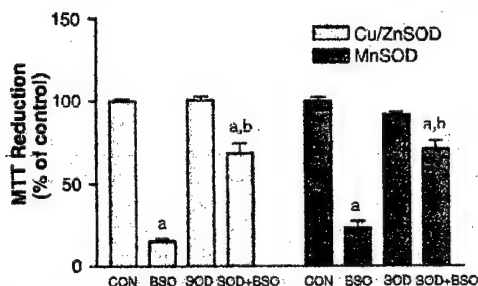
Fig. 7. Addition of SOD protects mesencephalic cultures from BSO toxicity. Cultures were exposed to 500 U/ml Cu/ZnSOD or 300 U/ml MnSOD with or without 50 µM BSO. MTT assay was performed 48 h after BSO treatment to determine cell viability. Bars show means ± S.E.M. ($n = 11$ for Cu/ZnSOD groups and $n = 8$ for MnSOD groups; for Cu/ZnSOD alone, $n = 7$); a differs from control, $P < 0.001$; b differs from BSO alone, $P < 0.001$. ANOVA followed by Tukey Kramer multiple comparisons test.

1996) and by the increased number of GFAP-positive astrocytic processes (Aschner, 1998). The pro-inflammatory cytokine IL-1β was also increased in microglia after treatment with LPS, a further indication of an activated state (Giulian et al., 1994).

In our culture system, LPS treatment did not cause apparent cell death or reduction in the uptake of dopamine, although it has been previously reported that LPS is selectively toxic to dopamine neurons in mesencephalic cultures (Bronstein et al., 1995). Differences in the cell culture conditions are likely the cause of this apparent discrepancy. However, in spite of lack of cell death, LPS treatment resulted in oxidative stress, which was implied by the reduction in GSH content in both astrocytes and mixed neuronal cultures. Furthermore, LPS potentiated the effect of BSO on GSH depletion, which suggests that the inflammatory response of glial cells causes oxidative

stress and has the potential to cause oxidative damage to the cultured cells. In addition, exposure of mesencephalic cultures to IL-1β increased BSO-induced damage, indicating that under conditions of oxidative stress IL-1β released by activated microglia could contribute to neurodegeneration. However, in spite of these apparently harmful effects, the overall result of LPS treatment was a significant protection from toxicity, suggesting that LPS may cause both pro- and antioxidant changes and that the protective events were predominant in our culture system.

Both astrocytes and microglia have the potential to provide support of neuronal survival *in vitro* (Engele et al., 1991; Hou et al., 1997; Nagata et al., 1993; O'Malley et al., 1992; Takeshima et al., 1994). Astrocytes stimulate neuronal growth, survival and regeneration by secretion of growth factors and extracellular matrix proteins (Fawcett, 1997; Muller et al., 1995). Following activation, astrocytes secrete both pro- and anti-inflammatory cytokines and growth factors (Aschner, 1998). We examined whether activated glial cells protected from damage caused by GSH depletion through the secretion of the growth factors bFGF, IGF-I and TGF-β. In a previous study we showed that bFGF reduced the damage caused by combined 6-hydroxydopamine and BSO treatment of mesencephalic cultures (Hou et al., 1997). In the present experiments bFGF, used at concentrations that produce maximum trophic effect in cultured neurons (Bouvier and Mytilineou, 1995), provided some protection from BSO toxicity when damage was not extensive, but it was substantially less effective than LPS. Therefore, it seems unlikely that secretion of bFGF by activated glia can by itself explain the protective effect of LPS treatment; although it may be contributory. TGF-β and IGF-I had no effect. LPS is a potent activator of IL-6 in astrocytes (Benveniste et al.,
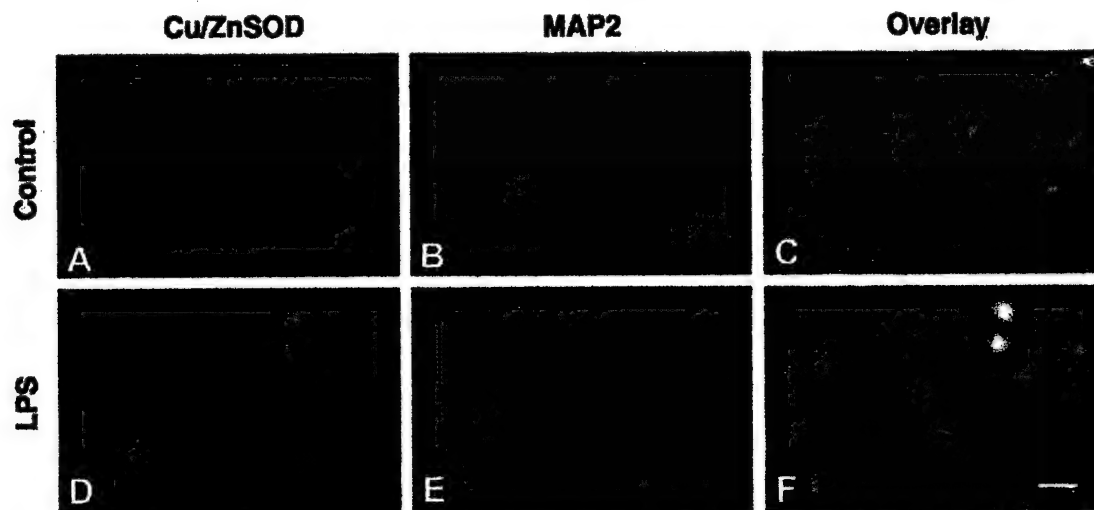

Fig. 8. Immunocytochemical localization of Cu/ZnSOD in mesencephalic cultures. Control (upper panel) and LPS-treated (10 µg/ml for 72 h; lower panel) cultures were double labeled for Cu/ZnSOD (A, D) and MAP-2 (B, E). Overlay of the two images (C, F) shows predominantly neuronal localization of Cu/ZnSOD and no apparent changes after treatment with LPS. Scale bar = 25 µm.
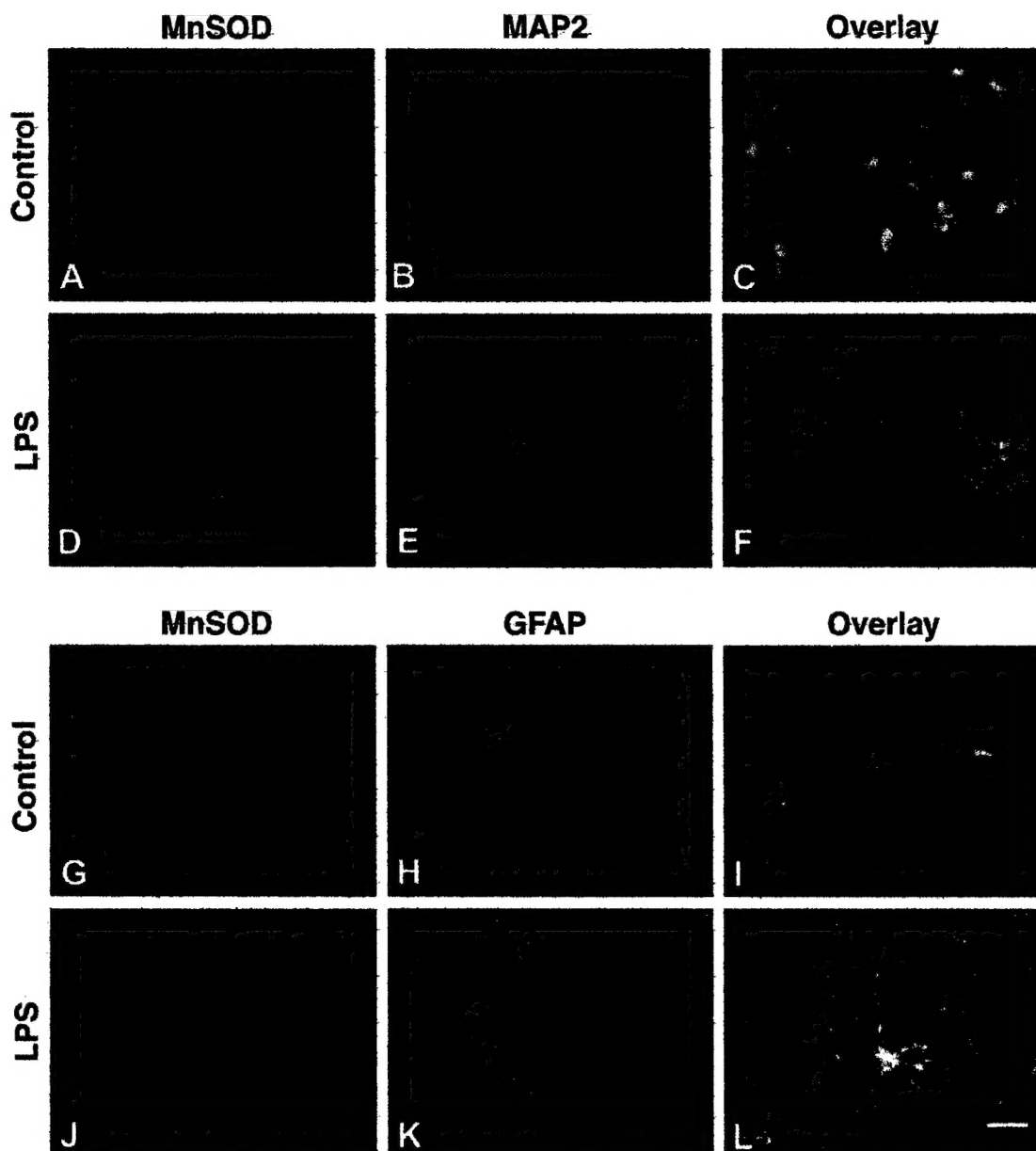
MnSOD	MAP2	Overlay

Fig. 9. Immunocytochemical localization of MnSOD in mesencephalic cultures. Control (first and third row) and LPS-treated (10 μg/ml for 72 h; second and fourth row) cultures were double labeled for MnSOD and MAP-2 (A–F) and MnSOD and GFAP (G–L). In control cultures MnSOD (A) was expressed strongly in neurons (B), as shown in the overlay of the two images (C). LPS treatment caused a very strong increase in MnSOD immunoreactivity (D), which did not co-localize with MAP-2-positive neurons (E, F). Double label with MnSOD (G) and GFAP (H) showed co-localization in some astrocytic processes (I). LPS treatment caused an increase in MnSOD (J), which was primarily within astrocytes (K, L). Scale bar = 25 μm.

1990) and IL-6 can have both trophic and toxic effects on neurons (Gruol and Nelson, 1997). However, in our experiments IL-6 was neither toxic, nor protective to GSH depleted cells. Similarly the cytokines IL-10, TNFα and INF-γ did not modify the damage caused by BSO treatment.

Compared to neurons, astrocytes are enriched in anti-oxidant enzymes (Desagher et al., 1996; Makar et al., 1994). Support of neuronal survival by astrocytes is believed to be due, in part, to the scavenging of extra-cellular reactive oxygen species (Drukarch et al., 1998; Peuchen et al., 1997). Recently, it has been shown that priming with small doses of LPS can protect mice against ischemia (Ahmed et al., 2000; Bordet et al., 2000;

Dawson et al., 1999; Tasaki et al., 1997). The beneficial effect paralleled the induction of inflammation and was attributed to a compensatory activation of SOD by LPS (Bordet et al., 2000). Exposure of neuronal and glial cell cultures to LPS also up-regulates MnSOD, the inducible form of SOD present in the mitochondria (Kifle et al., 1996; Yu et al., 1999). In our study we found that LPS caused a significant increase in both protein content and activity of MnSOD in mesencephalic cultures. The increase in MnSOD protein occurred primarily in GFAP-positive astrocytes. There was no change in the protein content or activity of Cu/ZnSOD, the constitutive form of the enzyme present in the cytoplasm. In addition, we found no change in catalase activity, in agreement with the results obtained by (Bordet et al., 2000), showing increased brain SOD activity but no changes in catalase after *in vivo* exposure to LPS. Activation of glial cells causes up-regulation and secretion of a number of pro- and anti-inflammatory cytokines, growth factors, as well as potential toxins (Minghetti and Levi, 1998), which makes it difficult to pinpoint at a single factor as the one responsible for the protective effect of LPS. However, a possible role for SOD in the LPS-induced protection was supported by the finding that addition of Cu/ZnSOD or MnSOD to mesencephalic cultures reduced the extent of BSO-induced damage. Both Cu/ZnSOD and MnSOD catalyze the dismutation of O_2^- and they would be expected to have a similar effect when added to the culture medium. Although the effect of exogenous SOD was likely extracellular, the protection observed probably resulted from scavenging of excessive O_2^- generated as a consequence of GSH depletion, which may have passed into the extracellular space. This is supported by a study showing that, in co-cultures of striatal glia and mesencephalic neurons, the depletion of GSH by BSO caused extracellular accumulation of ROS and cell loss, which was prevented by addition of SOD and catalase (Drukarch et al., 1998). Further experiments will be needed to identify the exact source of O_2^- in GSH depleted mesencephalic cultures, although damage by O_2^-, or its downstream ROS, would not necessarily be restricted to the cells of origin.

The selective increase in MnSOD in astrocytes compared to neurons is interesting and suggests that stimulation of astrocytes by LPS is likely involved in the up-regulation of MnSOD. It is also of interest that a selective increase in SOD within astrocytes results in the protection of all cells from oxidative damage. However, this concept is in agreement with numerous studies indicating that astrocytes can protect neurons from various oxidative insults (Desagher et al., 1996; Drukarch et al., 1998; Hou et al., 1997; Langeveld et al., 1995; Park and Mytilineou, 1992; Wilson, 1997).

There are several potential sources of O_2^- formation in cells, including oxidative phosphorylation used by the mitochondria for the generation of ATP. O_2^- is also formed during the metabolism of arachidonic acid by lipoxygenase, when hydroperoxyeicosetetraenoic acid (HPETE), the primary product of arachidonic acid metabolism, is converted to hydroxyeicosatetraenoic acid (HETE; Katsuki and Okuda, 1995). Our results suggest that O_2^- molecules play a very important role in the toxic events that follow GSH depletion. Merad-Saidoune et al. (1999) also reached a similar conclusion in a recent study showing that over-expression of MnSOD prevents mitochondrial damage caused by GSH depletion. Normally the concentration of O_2^- in the cells remains low as a result of the action of SOD. However, during GSH depletion SOD may not be able to handle the excess O_2^- generated from, among other sources, the metabolism of arachidonic acid by lipoxygenase. Arachidonic acid can also promote the generation of reactive oxygen species by directly inhibiting the mitochondrial respiratory chain (Cocco et al., 1999). When O_2^- levels become high, nitric oxide (NO) competes with SOD and combines rapidly with O_2^- to form peroxynitrite ($ONOO^-$). The damage to mitochondria caused by GSH depletion in neuronal and glial cultures has been shown to be the result of peroxynitrite production (Bolanos et al., 1995). Peroxynitrite can damage cells because it reacts with proteins acting as a selective oxidant and nitration agent (Bartosz, 1996). Nitrotyrosine is the product of the reaction of peroxynitrite with tyrosine and tyrosine residues (Reiter et al., 2000). The presence of nitrotyrosine in postmortem tissues in PD (Good et al., 1998) and other neurodegenerative disorders (Abe et al., 1995; Good et al., 1996; Sasaki et al., 2000), indicates that increased concentrations of O_2^- may contribute to their pathogenesis. Our data suggest that up-regulation of SOD activity may provide protection from oxidative stress and delay the progress of neurodegenerative diseases and in particular PD, where depletion of GSH may play a role in its pathogenesis (Jenner and Olanow, 1996; Sian et al., 1994).

Our study also illustrates the dependence of neurons on the surrounding glial cells. During conditions of oxidative stress, such as GSH depletion, the state of the surrounding glia can determine whether neurons will survive or die. Understanding of the relationships between neurons and glia should provide further insight into the process of neuronal degeneration, which contributes to the progression of neurological disorders.

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